Cell Reports, Volume *40*

Supplemental information

The IRAK4 scaffold integrates TLR4-driven TRIF and MYD88 signaling pathways

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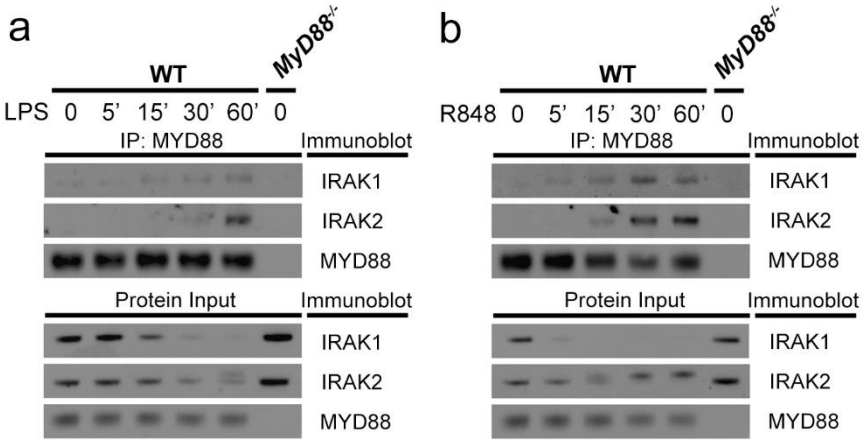

Figure S1 – MYD88 co-immunoprecipitation kinetics, Related to Figures 1, 2 and 4.

(a-b) Immunoblot analysis of MYD88 co-immunoprecipitation with IRAK1 and IRAK2 in WT BMDMs treated for up to 60 minutes with LPS **(a)** or R848 **(b)**. Stimulations were done with LPS 100 ng mL^{-1} or R848 1 μg mL^{-1}. Images are representative of three independent experiments.

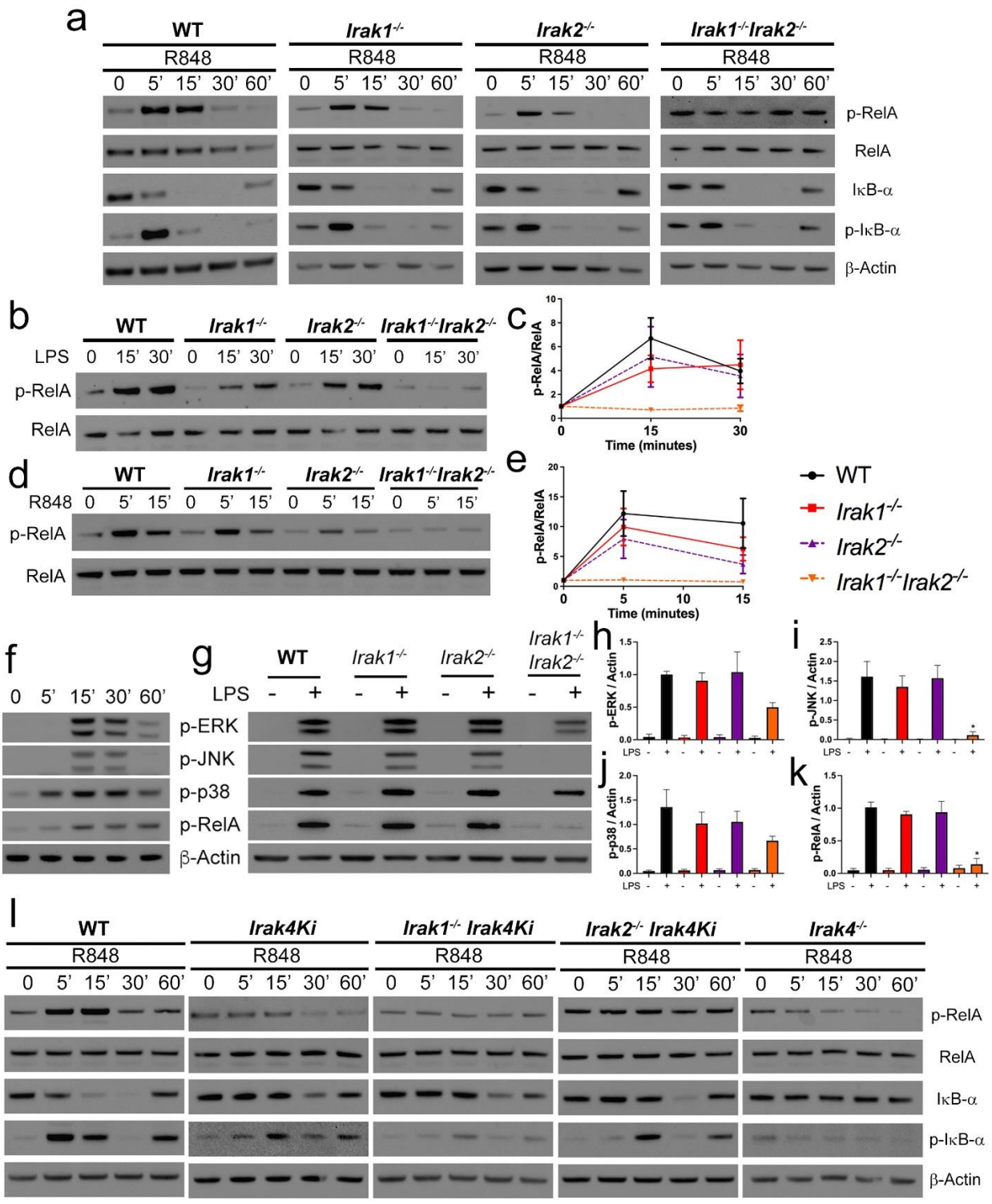

Figure S2 – NF-κB and MAPK activation in IRAK-deficient cells, Related to Figures 1 and 2.

(a) Kinetic study of p-RelA, RelA, IκB-α, p-IκB-α and β-actin by immunoblot of whole cell lysates from WT, *Irak1*$^{-/-}$, *Irak2*$^{-/-}$ and *Irak1*$^{-/-}$*Irak2*$^{-/-}$ BMDMs treated with R848 for up to 60 minutes. **(b-e)** RelA phosphorylation and densitometric quantifications in WT, *Irak1*$^{-/-}$, *Irak2*$^{-/-}$ and *Irak1*$^{-/-}$*Irak2*$^{-/-}$ BMDMs treated with LPS for up to 30 minutes **(b-c)** or R848 for up to 15 minutes **(d-e)**. **(f)** Kinetic study of p-ERK, p-JNK, p-p38, p-RelA and β-actin by

immunoblot of whole cell lysates from WT, BMDMs treated with LPS for up to 60 minutes. **(g-k)** ERK, JNK, p38 and RelA phosphorylation and densitometric quantifications in relation to β-actin in WT, *Irak1*$^{-/-}$, *Irak2*$^{-/-}$ and *Irak1*$^{-/-}$ *Irak2*$^{-/-}$ BMDMs unstimulated or stimulated with LPS for 15 minutes. **(l)** Kinetic study of p-RelA, RelA, IκB-α, p-IκB-α and β-actin by immunoblot of whole cell lysates from WT, *Irak4* Ki, *Irak1*$^{-/-}$*Irak4* Ki, *Irak2*$^{-/-}$*Irak4* Ki and *Irak4*$^{-/-}$ BMDMs treated with R848 for up to 60 minutes. All stimulations were done with LPS 100 ng mL^{-1} or R848 1 μg mL^{-1}. Images are representative of two **(f)**, three **(a, b, d, l),** or four **(g)** independent experiments. Data from three **(c,e)** or four **(h-k)** independent experiments (mean and s.e.m.).

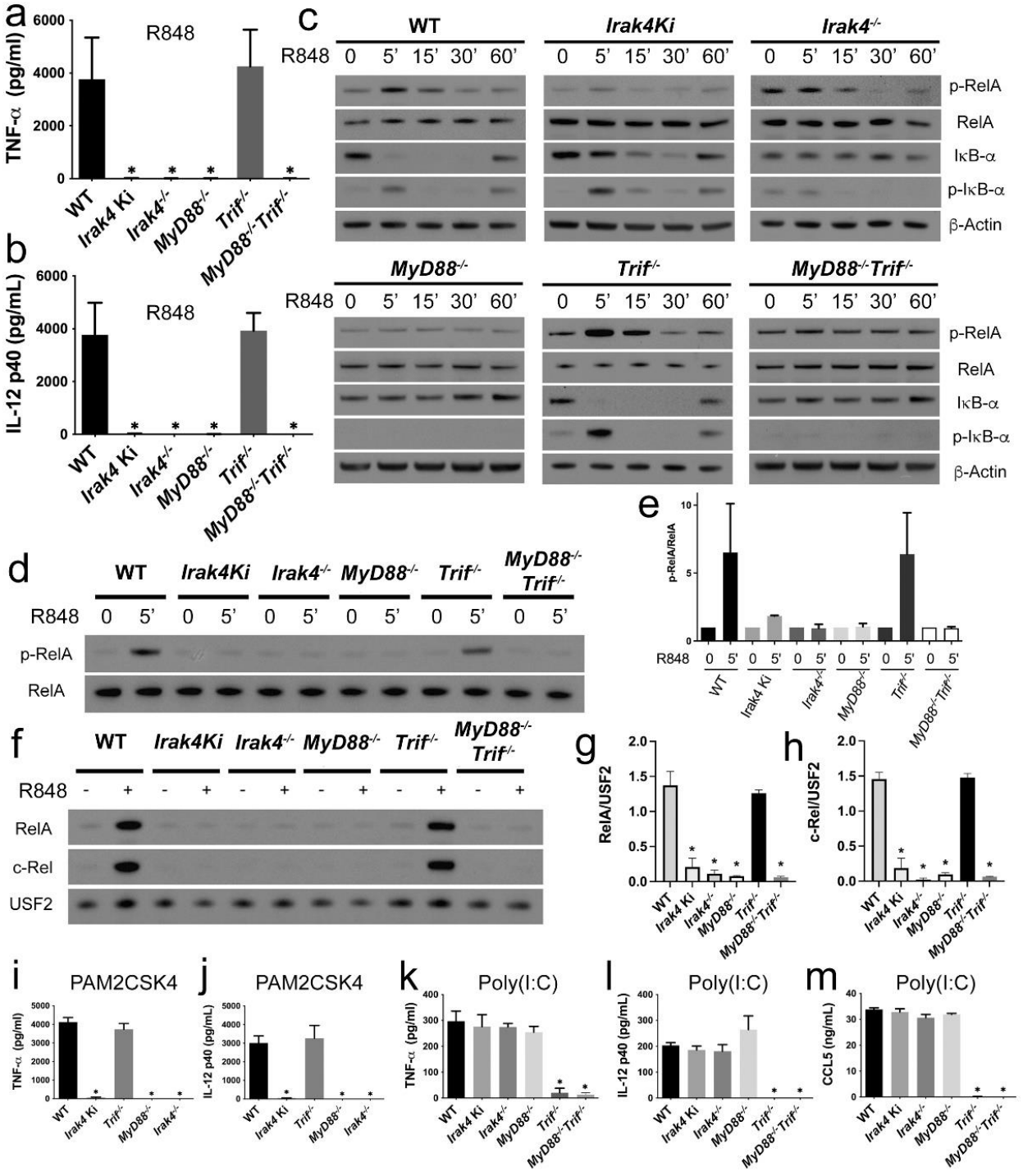

Figure S3 – TLR-2 and -7 signaling requires IRAK4 kinase activity, Related to Figure 3.

(a-h) Study of WT, *Irak4* Ki, *Irak4*$^{-/-}$, *MyD88*$^{-/-}$, *Trif*$^{-/-}$ and *MyD88*$^{-/-}$*Trif*$^{-/-}$ BMDMs stimulated with R848. **(a-b)** Quantification of TNF-α **(a)** and IL-12 **(b)** produced after R848 stimulation for 24 hours. **(c)** Kinetic study of p-RelA, RelA, IκB-α, p-IκB-α and β-actin by immunoblot of whole cell lysates from indicated BMDM strains stimulated with R848 for up to 60 minutes. **(d-e)** Immunoblot comparison p-RelA/RelA in indicated BMDM strains stimulated with R848 **(d)** and densitometric quantification **(e)**. **(f)** Immunoblot analysis of RelA and c-Rel in nuclear

lysates of WT, *Irak4* Ki, *Irak4*$^{-/-}$, *MyD88*$^{-/-}$, *Trif*$^{-/-}$ and *MyD88*$^{-/-}$*Trif*$^{-/-}$ BMDMs untreated or treated with R848 for 15 minutes and densitometric quantification of R848-treated samples **(g-h)**. **(i-j)** Quantification of TNF-α **(i)** and IL-12 **(j)** produced by WT, *Irak4* Ki, *Trif*$^{-/-}$, *MyD88*$^{-/-}$ and *Irak4*$^{-/-}$ BMDMs after stimulation with Pam3CSK4 for 24 hours. **(k-m)** Quantification of TNF-α **(k)**, IL-12 **(l)** and CCL5 **(m)** produced by WT, *Irak4* Ki, *Irak4*$^{-/-}$, *MyD88*$^{-/-}$, *Trif*$^{-/-}$ and *MyD88*$^{-/-}$*Trif*$^{-/-}$ BMDMs treated with Poly(I:C) (10 μg mL^{-1}) for 24 hours. All stimulations were done with R848 1 μg mL^{-1}, Pam3CSK4 200 ng mL^{-1}, or Poly(I:C) 10 μg mL^{-1} . *$p<0.05$ in comparison to WT (one-way analysis of variance with Tukey's multiple comparisons test). Data from three **(a-b, e, i-m)** or two **(g-h)** independent experiments (mean and s.e.m.). **(c-d, f)** Images are representative of two **(f)** or three **(c-d)** independent experiments.

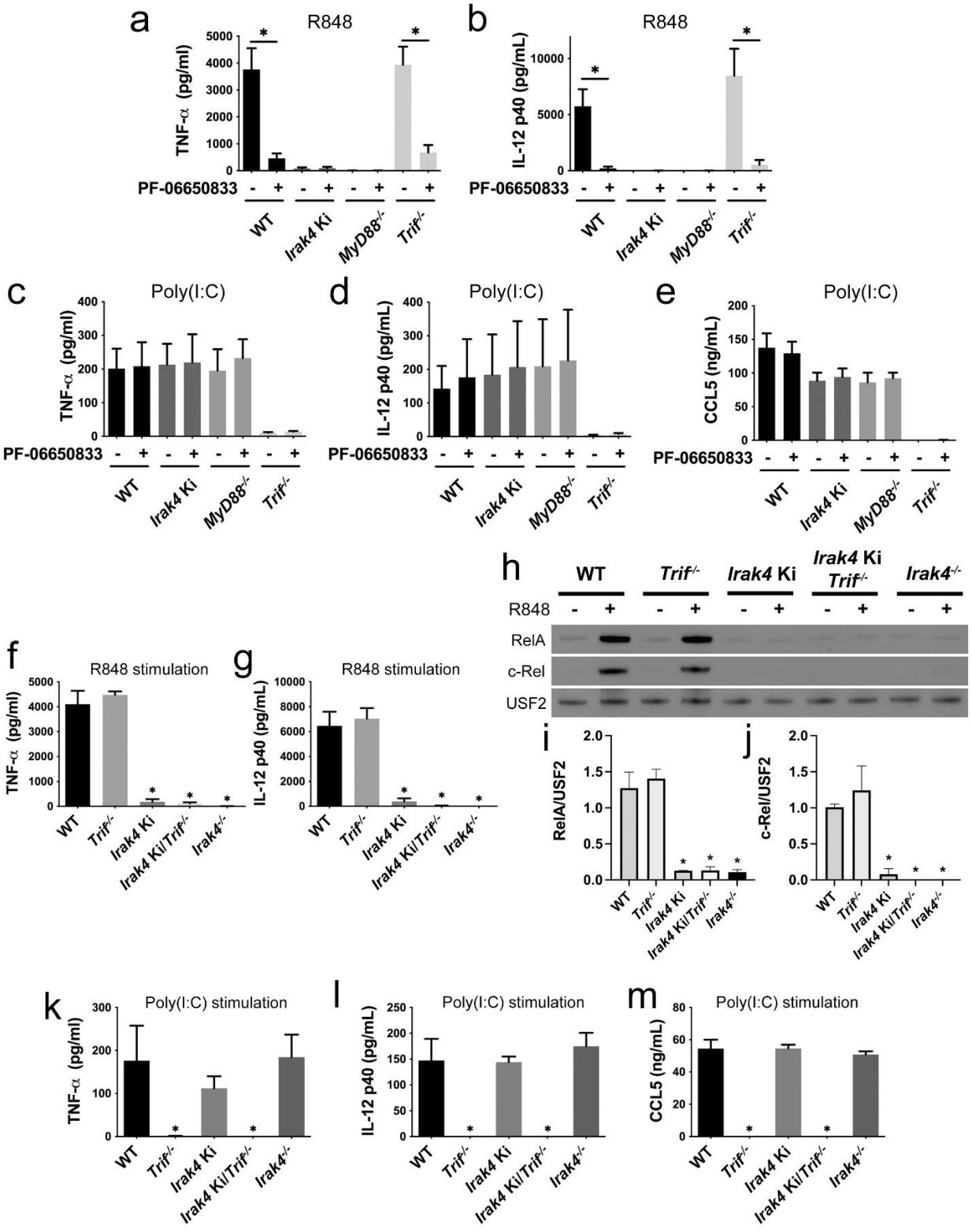

Figure S4 – IRAK4 inhibition affects cytokine production via TLR7 but not TLR3, Related to Figure 3 and 4.

(a-e) Quantification of TNF-α **(a,c)**, IL-12 **(b,d)** and CCL5 **(e)** produced by BMDMs treated with R848 **(a-b)** or Poly(I:C) **(c-e)** for 24 hours. **(f-m)** WT, *Irak4* Ki, *Trif*$^{-/-}$, *Irak4* Ki/*Trif*$^{-/-}$ and *Irak4*$^{-/-}$ BMDMs stimulated with R848

and Poly(I:C). **(f-g)** Quantification of TNF-α **(f)** and IL-12 **(g)** produced after stimulation with R848 for 24 hours. **(h)** Immunoblot analysis of RelA and c-Rel in nuclear lysates of WT, *Irak4* Ki, *Trif*$^{-/-}$, *Irak4* Ki/*Trif*$^{-/-}$ and *Irak4*$^{-/-}$ BMDMs untreated or treated with R848 for 15 minutes and densitometric quantification of R848-treated samples **(i-j)**. **(k-m)** Quantification of TNF-α **(f)**, IL-12 **(g)** and CCL5 **(h)** produced by WT, *Trif*$^{-/-}$, *Irak4* Ki, *Irak4* Ki/*Trif*$^{-/-}$ and *Irak4*$^{-/-}$ BMDMs treated with Poly(I:C) for 24 hours. All stimulations were done with R848 1 μg mL^{-1} or Poly(I:C) 10 μg mL^{-1}. *$p<0.05$ in comparison to WT unless indicated otherwise (one-way analysis of variance with Tukey's multiple comparisons test). Data from two **(i-j)** or three **(a-e, f-g, k-m)** independent experiments (mean and s.e.m.). **(h)** Images are representative of two independent experiments.

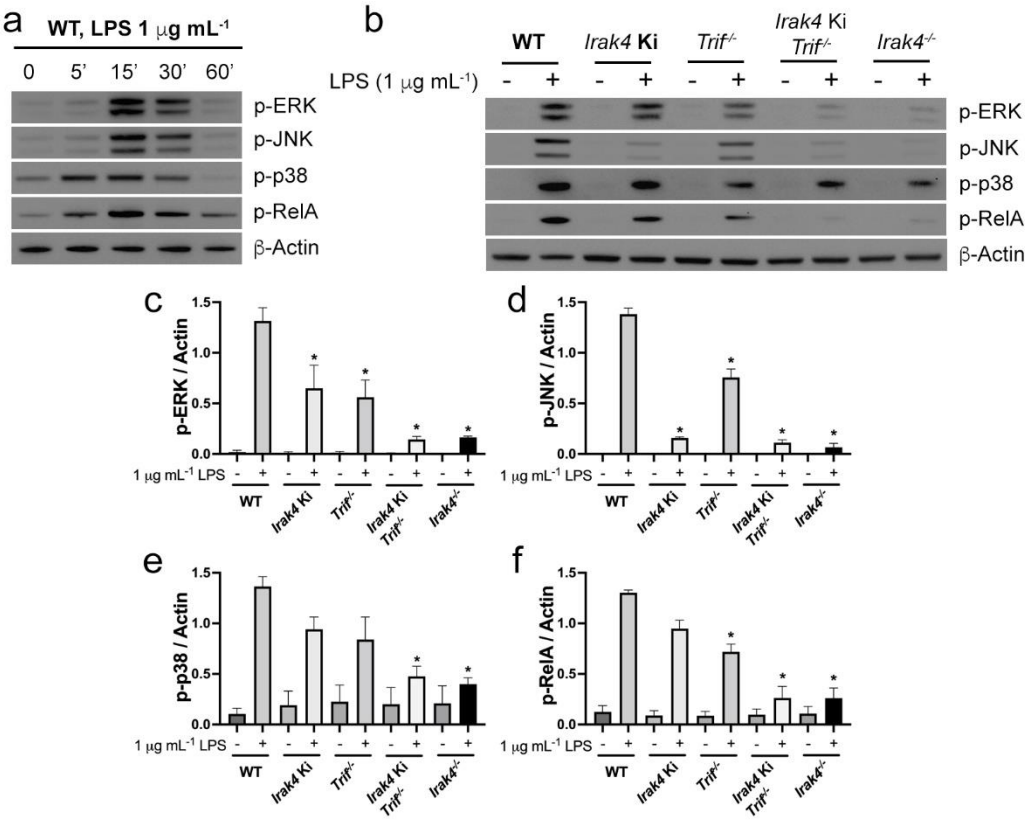

Figure S5 – MAPK and RelA activation in IRAK-deficient cells stimulated with 1 μg mL^{-1} of LPS, Related to Figure 4.

(a) Kinetic study of p-ERK, p-JNK, p-p38, p-RelA and β-actin by immunoblot of whole cell lysates from WT, BMDMs treated with LPS (1 μg mL^{-1}) for up to 60 minutes. **(b-f)** ERK, JNK, p38 and RelA phosphorylation and densitometric quantifications in relation to β-actin in WT, *Irak4* Ki, *Trif*$^{-/-}$, *Irak4* Ki, *Trif*$^{-/-}$, and *Irak4*$^{-/-}$ BMDMs unstimulated or stimulated with LPS (1 μg mL^{-1}) for 15 minutes. *p<0.05 in comparison to WT (one-way analysis of variance with Tukey's multiple comparisons test) **(a-b)** Images are representative of two **(a)** or three **(b)** independent experiments. **(c-f)** Data from three independent experiments (mean and s.e.m.).

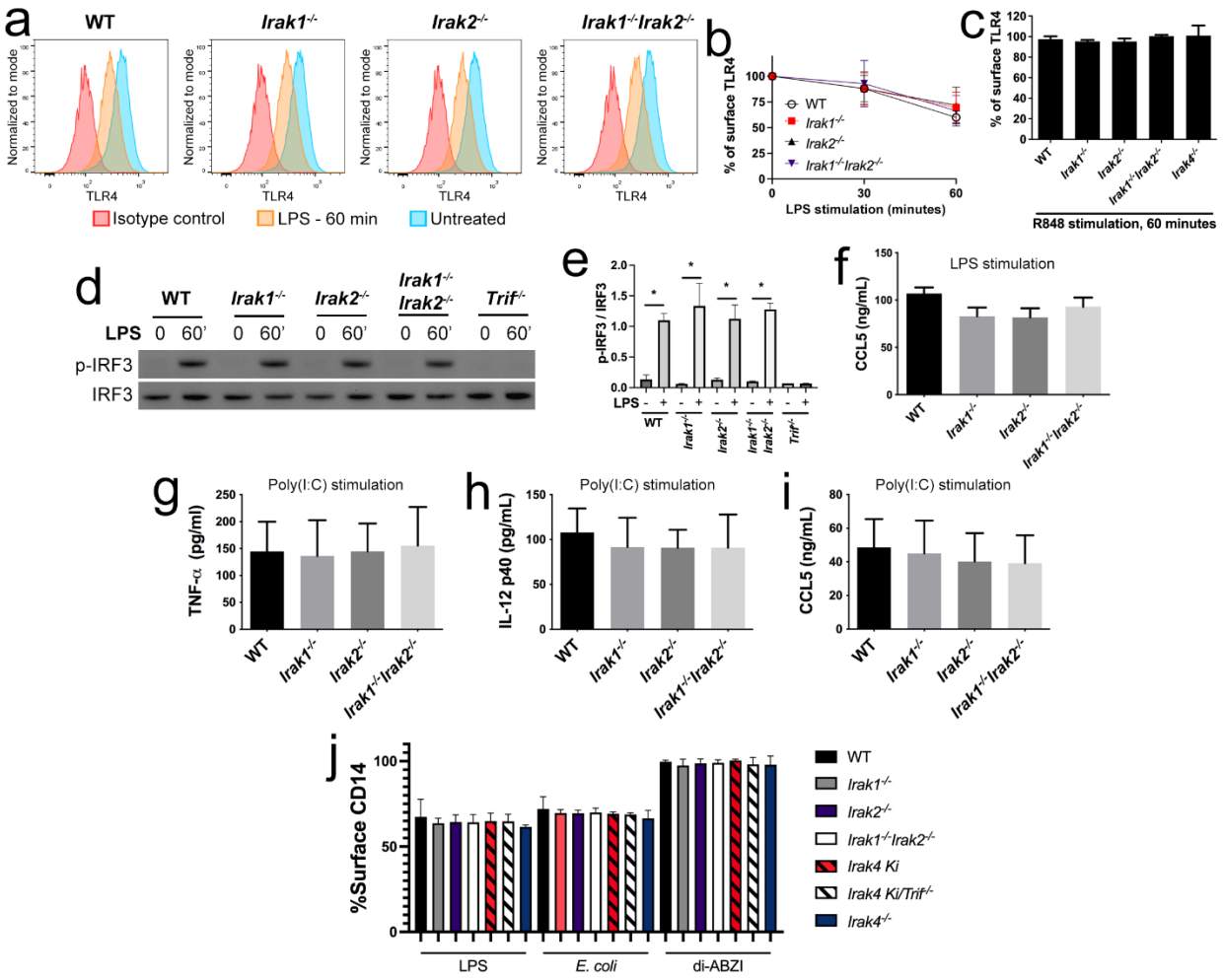

Figure S6 – IRAK1, IRAK2 and IRAK4 are not involved in TLR3- and TLR4-mediated TRIF signaling, Related to Figure 5.

(a-b) TLR4 endocytosis in WT, *Irak1*$^{-/-}$, *Irak2*$^{-/-}$ and *Irak1*$^{-/-}$*Irak2*$^{-/-}$ BMDMs stimulated with LPS for up to 60 minutes. **(a)** Histogram of surface TLR4 in the indicated strains unstimulated, stimulated with LPS for 60 minutes, or unstimulated cells stained with isotype control antibody. **(b)** Quantification of surface TLR4 after LPS stimulation for up to 60 minutes. **(c)** Quantification of surface TLR4 in BMDMs stimulated with R848 (used here as negative control for TLR4 endocytosis) for 60 minutes. **(d)** IRF3 phosphorylation of whole cell lysates from WT, *Irak1*$^{-/-}$, *Irak2*$^{-/-}$ and *Irak1*$^{-/-}$*Irak2*$^{-/-}$ BMDMs and **(e)** densitometric analysis. **(f)** Quantification of CCL5 produced by WT, *Irak1*$^{-/-}$, *Irak2*$^{-/-}$ and *Irak1*$^{-/-}$*Irak2*$^{-/-}$ BMDMs after stimulation with LPS for 24 hours. **(g-i)** Quantification of TNF-α **(g)**, IL-12 **(h)** and CCL5 **(i)** produced by WT, *Irak1*$^{-/-}$, *Irak2*$^{-/-}$ and *Irak1*$^{-/-}$*Irak2*$^{-/-}$ BMDMs treated with Poly(I:C) for 24 hours. **(j)** CD14 endocytosis in WT or IRAK-deficient BMDMs stimulated for 30 minutes with LPS, E. coli Bort, or di-ABZI (negative control). All stimulations were done with LPS 100 ng mL^{-1}, R848 1 μg mL^{-1}, Poly(I:C) 10 μg mL^{-1}, E. coli Bort MOI 1 and di-ABZI. 1 μg mL^{-1}. *p<0.05 (one-way analysis of variance with Tukey's multiple comparisons test) **(a)** Data is representative of three independent experiments. **(d)** Images are representative of two independent experiments. Data from two **(e)** or three **(b-c, f-j)** independent experiments (mean and s.e.m.).

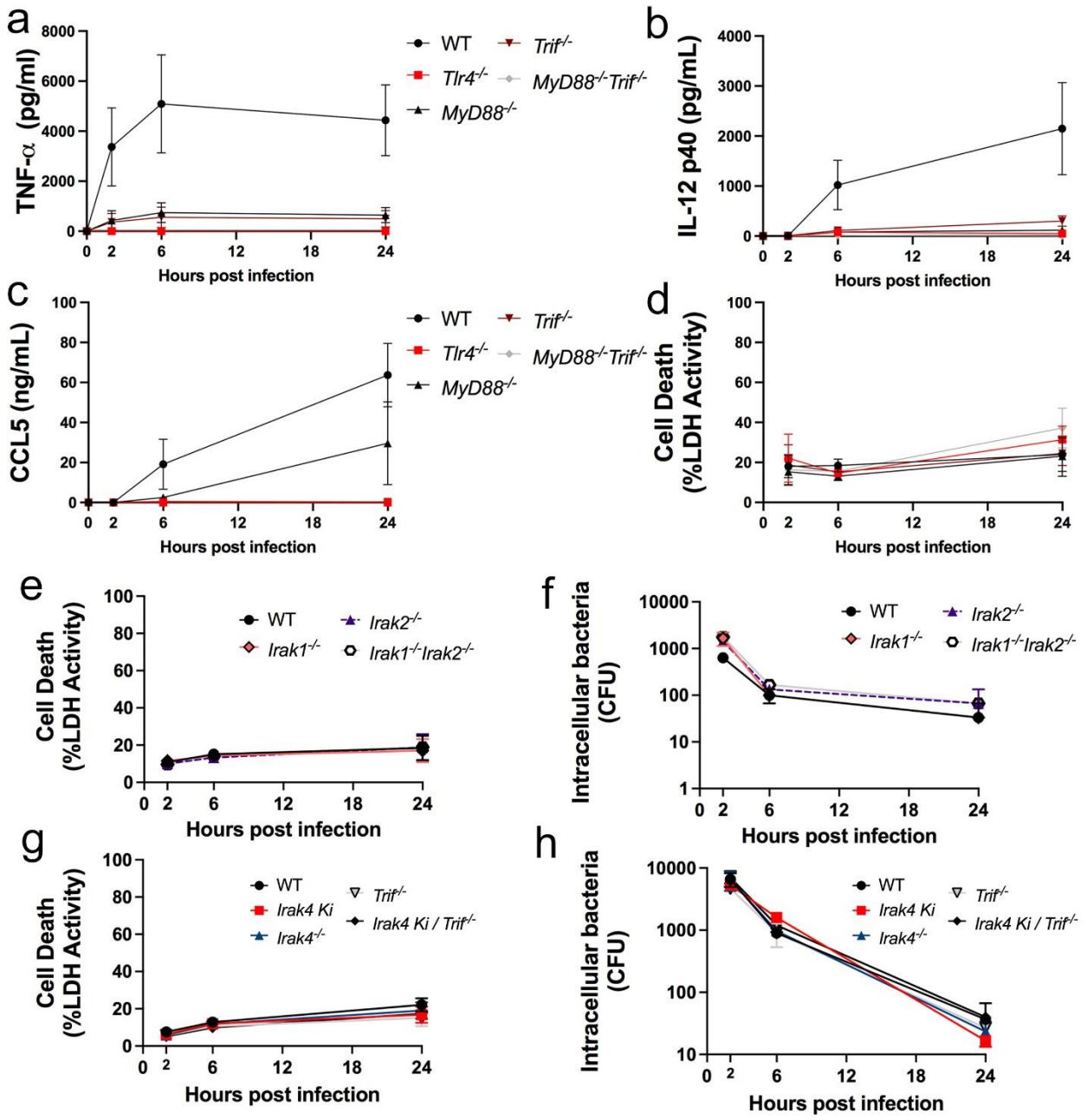

Figure S7 – Cytokine production and cell viability in response to *E. coli* Bort, Related to Figure 7.

(a-d) Production of TNF-α **(a)**, IL-12 **(b)** CCL5 **(c),** and LDH release **(d)** in WT, *Tlr4*$^{-/-}$, *MyD88*$^{-/-}$, *Trif*$^{-/-}$ and *MyD88*$^{-/-}$*Trif*$^{-/-}$ BMDMs infected with *E. coli* Bort at MOI 1 for up to 24 hours. **(e-f)** LDH release **(e)** and viable intracellular bacteria **(f)** in WT, *Irak1*$^{-/-}$, *Irak2*$^{-/-}$ and *Irak1*$^{-/-}$*Irak2*$^{-/-}$ BMDMs infected with *E. coli* Bort for up to 24 hours at MOI 1. **(g-h)** LDH release (**g**) and viable intracellular bacteria **(h)** in WT, *Irak4 Ki*, *Irak4*$^{-/-}$, *Trif*$^{-/-}$ and *Irak4* Ki/*Trif*$^{-/-}$ BMDMs infected with *E. coli* Bort for up to 24 hours at MOI 1. **(a-b, d-e)** Data from three independent experiments (mean and s.e.m.). Data from two **(f, h)** or three **(a-d, e, g)** independent experiments (mean and s.e.m.).